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Molecular identification and screening of mushrooms for antibacterial property against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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Abstract
In this study, 18 mushrooms were collected from Gandhi Krishi Vigyana Kendra (GKVK) campus, University of Agricultural Sciences, Bangalore (India) and identified by Internal Transcribed Spacer (ITS) region using National Centre for Biotechnology Information (NCBI) data base and screened for antibacterial property against two skin infection causing bacteria *viz.,Pseudomonas aeruginosa* and *Staphylococcus aureus* in dogs. Eleven mushrooms extract inhibited both the bacteria showing their antimicrobials as broad spectrum while inhibiting gram positive as well as gram negative bacteria. The highest zone of inhibition was recorded from the extract of *Lyophyllum sp.* The extract of two mushrooms viz., *Phlebopus portentosus* and *Termitomyces* sp. inhibited only *P. aeruginosa*, a gram negative bacterium and the extract of three mushrooms viz., *Agaricus* sp, *Coprinellus disseminates* and *Agaricus blazei* inhibited only *S. aureus*, a gram positive bacterium, suggesting spectral specificity of their antimicrobial compounds. However, the extracts of two mushrooms *viz.,Macrocybe gigantea* and *Schizophyllum commune* did not show inhibition of any of the pathogens. Hence, this study suggests that the occurrence of potential pharmaceuticals in mushrooms can be exploited for control of multidrug resistant bacteria.

Keywords: Antibacterial activity, ITS region, Mushrooms, *P. aeruginosa, S. aureus*

INTRODUCTION

Mushrooms are macro-fungi with a distinctive fruiting body, that can be either hypogeous or epigeous, large enough to be seen by the naked eye and to be picked up by hand (Chang and Miles, 1992). Mushrooms produce antibacterial and antifungal compounds to survive in their natural environment. It is therefore not surprising that antimicrobial compounds with more or less strong activities could be isolated from mycelia or fruiting body which are useful for human health (Lindequist *et al*., 1990). Mushrooms are superior nutritional supplement but attributed with magnificent medicinal values too. The bioactive compounds present in mushroom includes, polysaccharides, lipopolysaccharides, proteins, peptides, glycoproteins, nucleosides, triterpenoids, lectins, lipids and their derivatives (Sharma *et al.,* 2014). Fruiting bodies, mycelia, and spores accumulate a variety of bioactive metabolites with immunomodulatory, cardiovascular, liver protective, antifibrotic, anti-inflammatory, antidiabetic, antiviral, antioxidant, antitumor, and antimicrobial properties. *Staphylococcus aureus* is a Gram-positive coccus that are commonly found on the skin of most mammals. These bacteria are able to produce and secrete numerous cell products that cause severe tissue damage. *Staphylococci* are also particularly adept at avoiding the effects of antibiotics. In particular, methicillin-resistant Staphylococci have emerged as a serious medical problem and they are able to develop and transfer resistance to numerous antibiotics by means of several divergent mechanisms (Upadhyay, 2011)

Pseudomonas aeruginosa is an opportunistic Gram negative pathogen of both human and veterinary species. This bacterium almost never affects healthy tissue but prefers damaged or diseased regions of the skin. In dogs, *Pseudomonas* otitis is the principal disease process caused by this organism. It is likely that the chronic alterations in the otic microenvironment associated with persistent inflammation, sets up a state of hyperhidrosis. The unique pathogenicity of *Pseudomonas* allows it to take advantage of the diseased tissue and develop resistance to antibiotics (Cholley *et*

al., 2010).

The drug resistant patterns of the pathogens in community health settings are worrisome as most of these resistant bacteria are capable of horizontal gene transfer, decreased cell permeability against conventional antibiotics, and alteration of the ribosomal binding site (Adwan *et al*., 2014). Thus, the problem of antibiotic resistance is now become a global challenge in both under developed and developing countries (Levy, 2002; Sosa *et al*., 2010). The bacterial pathogens, *P. aeruginosa* and *S. aureus* are causing skin infection in animals and they are posing resistant to chemically synthesized antibiotics. In order to curb the problem of antibiotic resistances, the use of biologically active compounds originated from plants and fungi are necessary. The health-promoting benefits of secondary metabolites sourced from medicinal mushrooms had tremendously increased and the chances of obtaining novel and safe antimicrobial compounds, which would combat and reduce the incidence of antibiotic resistance (Alves *et.al*., 2012). Therefore, we attempted to explore the antibacterial mushrooms against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

MATERIALS AND METHODS

Molecular identification using ITS region sequence: During rainy season (July-September 2016), fruiting bodies of 18 wild mushrooms were collected from different locations at GKVK (Gandhi Krishi Vigyana Kendra) campus, University of Agricultural Sciences, Bengaluru-560065. The genomic DNA was extracted from tissue by modified CTAB (Cetyl Trimethyl Ammonium Bromide) lysis method (Doyle and Doyle, 1987). Fruiting body (0.1 g) was ground with extraction buffer (1.5 ml) containing CTAB, mixed with PVP and ß mercapto ethanol and incubated at 65 °C for 45 minutes. Half of the sample was transferred into a new tube and equal volume of chloroform: isoamyl alcohol (24:1) was added. They were centrifuged at 10,000 rpm for 10 minutes and the supernatant was transferred into a new tube. The DNA was precipitated by adding 600 µl of chilled isopropanol and centrifuged at 10,000 rpm after overnight incubation. The pellet was washed with of 70 % chilled ethanol, air dried and dissolved in 20-25 µl of double distilled water. The Ribo Nucleic Acid (RNA) was removed by adding 5 µl of RNase and incubated at 37 °C for one hour. The purity and concentration the DNA was checked at 260 nm using BioMATE 3S UV-Visible spectrophotometer (Thermo Fisher Scientific, USA).

Genomic DNA was PCR amplified using 40 µl reaction mixture containing 4.0 µl 10x Taq. Buffer, 4.0 µl 10mM dNTP mixture, 2.0 µl ITS Primers, 0.6 µl Taq. DNA polymerase, 2.0 µl Template DNA, 27.4 µl sterile distilled water (Sambrook *et al*., 1989). The PCR was carried out in a thermal cycler programmed as follows. Initial denaturation at 94 °C for 4 min. 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 30 secs, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The amplified products were separated by agarose gel (1 %) electrophoresis. Then the DNA was eluted from the agarose gel using gel extraction kit (Gene JET™ Gel Extraction Kit,Thermo Scientific) and the eluted DNA was got sequenced by Scigenom Labs Pvt. Ltd, India using ITS1 and ITS4 primers. Sequence results were analyzed with online software (*http:// www.ncbi.nlm.nih.gov/BLAST*) of National Centre for Biotechnology (NCBI) (Altschul *et al*., 1990).

Preparation of mushroom extract and screening for antibacterial property: One gram of fresh tissue from the fruiting body was washed, surface sterilized using 5% Sodium hypochlorite solution and repeatedly washed with sterile water. The tissue was crushed in 5ml sterile water using pestle and mortar to obtain 20 % aqueous extract (1g in 5 ml). The extract was centrifuged and supernatant was collected. The extract thus obtained was filter sterilized using 0.22 µ membrane filters attached to sterile disposable syringes. The extracts were collected in sterile eppendorff tubes and stored at -20 °C (Blue star, CHF 100B). The sterile water was used as control. The two bacteria *viz., P. aeruginosa* and *S. aureus* isolated from dog's otitis infection and dermatitis (Fig.1) from the Institute of Animal Health and Veterinary Biologicals (IAH abd VB), Karnataka Veterinary, Animal and Fisheries Sciences University (KVAFSU), Bangalore-560024 were obtained and cultured on Brain Heart Infusion Agar and Mannitol Salt Agar respectively.

The bacterial lawn was prepared on solidified nutrient agar medium. Sterile filter paper discs (Whatman No. 42) of 5 mm diameter were dipped in 20 % mushroom extracts and placed on the lawn of the test organisms. Three filter paper discs have been used for each mushroom extract. The filter paper disc dipped in sterile water was used as control on the lawn. These plates were incubated at 37 °C. The observation for inhibition zone was recorded after 24 hours. The minimum inhibitory concentration was determined by using different concentrations.

RESULTS AND DISCUSSION

Molecular identification: Mushrooms can be identified by both phenotypic and molecular characters. The morphological characters alone used in the classification are time consuming and often inadequate for exact strain identification. Morphological characterization also needs basic knowledge on the structure or phenotypic characters of mushrooms (Arora, 1986). However, in recent years, the molecular approach by using

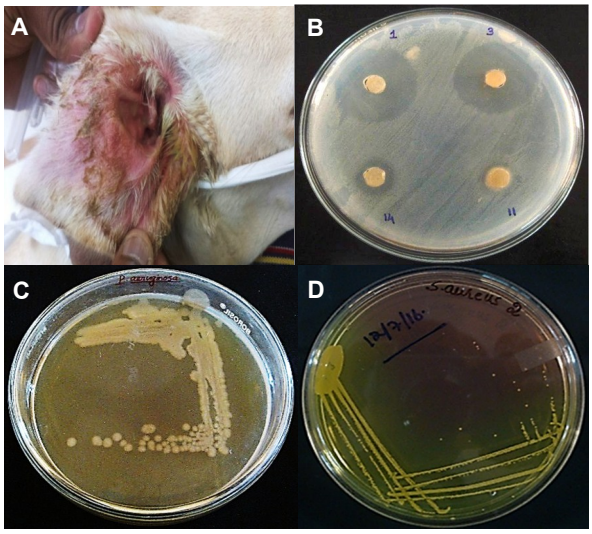

Fig.1.A. *Dog's ear infection. B. Inhibition zone aused by mushroom extract C. Colonies of P. aeruginosa. D. Colonies of S. aureus.*

Internal Transcribed Spacer (ITS) region/ 18S rRNA gene sequence are used for identification of mushrooms including other eukaryotes, as these sequences are conserved irrespective of life history and evolution (Rajaratnam and Thiagarajan, 2012). The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) has been widely used for identification of mushrooms at both species and genus level (Sanchez-Ballesteros *et al.,* 2000). The ITS region fragment ranges between 450 to 700 base pair has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined gap

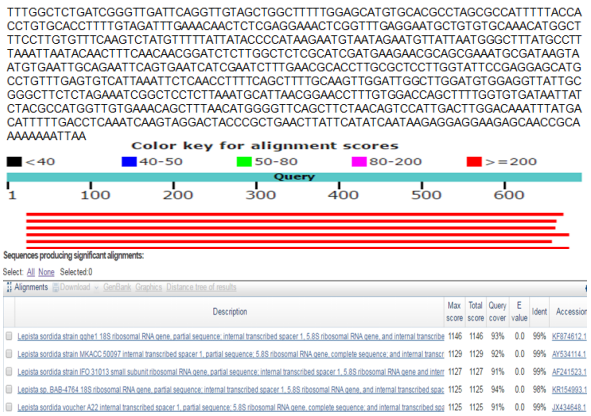

Fig.2. *Partial sequence length and homology search of Lepista sordida (GKVK-1)*

between inter- and intra-specific variation (Schoch *et al.,* 2012). In the present study, eighteen mushrooms collected from different location of the campus were designated as GKVK-1, GKVK-2, GKVK-3, GKVK-4, GKVK-5, GKVK-6, GKVK-7, GKVK-8, GKVK-9, GKVK-10, GKVK-11, GKVK-12, GKVK-13, GKVK-14, GKVK-15, GKVK-16, GKVK-17 and GKVK-18 were identified up to species level by using ITS rRNA region sequence. The PCR amplification of genomic DNA of the eighteen mushrooms in the present study yielded amplified product size varying from 440 to 1054 bp which are corresponding to almost full length gene sequence of ITS rRNA. The sequence homology of the eighteen species ranged from 87-99 per cent when aligned with the sequences present in NCBI (*http://www.ncbi.nlm.nih.gov/BLAST*) (Altschul *et al.*, 1990). The mushroom designated as GKVK-1

Table 1. Effect of aqueous extract of mushrooms on *P. aeruginosa* and *S. aureus.*

Mushrooms	**Diameter of inhibition zone (mm)**	
	P. aeruginosa	***S. aureus***
Control (sterile water)	0.00^{f}	0.00^{g}
Lepista sordida	7.33^{a}	8.50^{a}
Lyophyllum sp.	6.17^{de}	5.83^{ef}
Psathyrella candolleana	7.17^{ab}	7.50^{bc}
Lepiota sp.	6.67^{bcd}	6.50^{def}
Macrocybe gigantea	0.00^{f}	0.00^{g}
Agaricus xanthodermus	6.17^{de}	7.50^{bc}
Agaricus sp.	6.17^{de}	7.67^{ab}
Hymenagaricus sp.	6.83^{abc}	6.17^{ef}
Ganoderma multipileum	7.33^{a}	6.67^{cde}
Agaricus sp.	0.00^{f}	7.33^{bcd}
Schizophyllum commune	0.00^{f}	0.00^{g}
Agrocybe pediades	6.17^{de}	6.67^{cde}
Phlebopus portentosus	6.00^{e}	0.00^{g}
Omphalotus olivascens	6.33^{cde}	7.17^{bcd}
Coprinellus disseminatus	0.00^{f}	7.33^{bcd}
Termitomyces sp.	6.67^{bcd}	0.00^{g}
Geastrum sp.	6.33^{cde}	6.50^{def}
Agaricus blazei	0.00^{f}	5.67^{f}
S. Em±	0.187	0.296
LSD @ 5 %	0.536	0.848

Means of the same superscript in a column do not differ significantly @ P = <0.05 as per Duncan Multiple Range Test

Fig. 3. *Mushrooms identified and screened for antibacterial property. A- Lepista sordida, B- Lyophyllum sp., C- Psathyrella candolleana, D- Lepiota sp., E- Macrocybe gigantea, F- Agaricus xanthodermus, G- Agaricus sp., H- Hymenagaricus sp., I- Ganoderma multipileum, J- Agaricus sp., K- Schizophyllum commune, L- Agrocybe pediades, M- Phlebopus portentosus, N- Omphalotus olivascens, O- Coprinellus disseminatus, P- Termitomyces sp., Q-Geastrum sp., R- Agaricus blazei*

showed 99 percent homology to *Lepista sordida* (Fig.2), GKVK-2 with 98% to *Lyophyllum* sp., GKVK-3 with 95% to *Psathyrella candolleana,* GKVK-4 with 95% to *Lepiota* sp., GKVK-5 with 99% to *Macrocybe gigantean,* GKVK-6 with 97% to *Agaricus xanthodermus,*GKVK-7 with 99% to *Agaricus* sp., GKVK-8 with 87% to *Hymenagaricus* sp., GKVK-9 with 99% to *Ganoderma multipileum,* GKVK-10 with 95% to *Agaricus* sp., GKVK-11 with 98% to *Schizophyllum commune,* GKVK-12 with 99% to *Agrocybe pediades,* GKVK-13 with 99% to *Phlebopus portentosus,* GKVK-14 with 99% to *Omphalotus olivascens,*GKVK-15 with 98% to *Coprinellus disseminates,* GKVK-16 with 98% to *Termitomyces* sp., GKVK-17 with 97% to *Geastrum* sp., and the GKVK-18 has 99% homology with *Agaricus blazei* (Fig.3, A-R, shows the fruit bodies of 18 identified mushrooms). Identification of mushrooms using ITS region sequence is advantageous as organism can be identified at any stages of the growth and it is widely used for identification several fungi (Schoch *et. al*., 2012). Raju *et al*. (2014) identified *Volveriella* species by PCR amplification of ITS region using ITS 1 and ITS 4 primers. Similarly, fourteen mushroom species including *Termitomyces* documented from Biligiri Rangana Hills of Westernghats of Karnataka were identified by ITS region sequence (Akash *et al*., 2017).

Effect of mushrooms extract on pathogens: Antimicrobial property of mushrooms due to the presence of antibiotics, tannins, alkaloids, flavonoids etc.(Draughon,2004). Out of eighteen wild mushroom extracts screened, eleven showed effective in inhibiting both the bacteria (Table,1). Significantly highest zone of inhibition was observed for *Lepista sordida.* The diameter of inhibition zone of this mushroom was 7.33 mm and 8.50 mm for *P aeruginosa* and *S aureus* respectively. The next best was the *Psathyrella candolleana* which caused 7.17 mm and 7.50 mm diameter of inhibition zone for *P. aeuruginosa* and *S*

Table 2. Minimum inhibitory concentration of mushroom extracts against *P. aeruginosa*

Mushrooms	Per cent concentration															
	20	18	16	14	12	10	8	6	4	2	1	0.8	0.6	0.4	0.2	0.09
Lepista sordida	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Lyophyllum sp.	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Psathyrella candolleana	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Lepiota sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Agaricus xanthodermus	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Agaricus sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Hymenagaricus sp.	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Ganoderma multipileum	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
Agaricus sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Agrocybe pediades	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Omphalotus olivascens	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
Coprinellus disseminatus	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Geastrum sp.	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Agaricus blazei	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = positive for inhibition zone; - = negative for inhibition zone

Table 3. Minimum inhibitory concentration of mushroom extracts on *S. aureus.*

Mushrooms	Per cent concentration														
	20	18	16	14	12	10	8	6	4	2	1	0.8	0.6	0.4	0.2
Lepista sordida	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Lyophyllum sp.	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Psathyrella candolleana	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Lepiota sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Agaricus xanthodermus	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Agaricus sp.	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Hymenagaricus sp.	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
Ganoderma multipileum	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Agrocybe pediades	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Phlebopus portentosus	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Omphalotus olivascens	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
Termitomyces sp.	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
Geastrum sp.	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-

+ = positive for inhibition zone: - = negative for inhibition zone

aureus respectively. Rest of the mushroom species showed varied inhibition zone for each pathogen. This indicates that these mushrooms in general contain antibacterial compounds that are probably broad spectrum ones and efficacy varied with mushroom species. The variations in the antimicrobial activities of mushrooms may be due to the differences in their bioactive compositions or concentrations, methods of extraction and mechanism of action of active ingredients present in these mushrooms (Iwalokun *et al.,* 2007). Further, the extracts of two mushrooms (*Phlebopus portentosus* and *Termitomyces* sp.) inhibited only *P. aeruginosa* and three mushrooms viz., *Agaraicus* sp*., Coprinellus disseminates* and *Agaricus blazei* inhibited only *S. aureus.* This variation in antimicrobial activities of mushrooms may be due the differences in their bioactive compositions or concentrations, methods of extraction and mechanism of action of anti-ingredients in mushrooms (Iwalokun *et al*., 2007) as *P. aeruginosa* is a gram negative and *S. aureus* is a gram positive bacteria. Quang *et al*. (2006) reported that the benzoic acid derivative isolated from mushroom *Xylaria intracoiarata* showed antibacterial activity against only gram negative bacteria viz., *Escherichia coli, Psiudomonas aeuroginosa* and *Solmonella enteritidis*.

Two mushrooms *viz., Macrocybe gigantea* and *Schizophyllum commune* did not show any inhibition on either bacterium. These mushrooms might not have produce antibacterial against the targeted pathogens. Otherwise, method of extraction may also influence because, in earlier studies, ethanol extract of *Ganoderma lucidum* did not inhibit *Staphylococcus aureus, Bacillus, Alcaligenes faecalis,* and *Proteus vulgaris* but, benzene, chloroform, and ethyl acetate extracts gave better results on inhibition of test organisms (Shikongo *et al.* 2013). Beattie *et al.* (2010) also reported the efficacy of ethyl acetate extract of the mushrooms viz., *Cortinarius ardesiacus, C. archeri, C. atrosanguineus, C. austrovenetus, C. austroviolaceus, C. coelopus, C. clelandii, C. memoria -annae, C. persplendidus, C. sinapicolor, C. vinosipes* on inhibition of *Staphylococcus aureus.*

The minimal inhibitory concentration (MIC) of mushrooms extract varied between two pathogens. MIC ranged between 0.2% to 16% for *P. aeruginosa* (Table,2, Fig.1B). Least concentration

of *L. sordida* extract being 2 percent showed inhibition of *P. aeruginosa* indicating its potentiality at minimal concentration. Similarly, the extract of mushrooms *P. candolleana* and *Lepiota* sp. showed 0.4% as MIC against *S. aureus* (Table,3). This suggest that the antimicrobial present in different mushrooms and their mode of action may differ for different pathogen. Kolundzic and Marina (2016) reported that the antibacterial activity of aqueous extracts of tinder fungus *Fomes fomentarius* against *Helicobacter pylori* with MIC values between 4-30 µg/ml.

Conclusion

In our study, 16 mushroom species exhibited antibacterial activity against *P. aeruginosa* and *S. aeurus*. Though the inhibition pattern varied for different mushroom species, all 16 mushrooms can be exploited for isolation of pure compounds to use against multi drug resistant pathogens as only crude extracts were used in this study. Among 16 mushroom species, the *L. sordida* showed maximum inhibition zone against both bacteria. Therefore, *L. sordida* would be the best mushroom for antibacterial compounds compared to others. Further, *P. portentosus* and *Termitomyces* sp can be exploited for controlling gram negative bacteria as they specifically inhibited *P. aeruginosa* but not *S aureus.* Similarly, *Agaricus* sp, *Agaricus blazei* and *Coprinellus disseminates* were more efficient against gram positive bacteria as they inhibited *S. aureus* alone.

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